The stability, toxicity and effectiveness of unmodified and phosphorothioate antisense oligodeoxynucleotides in Xenopus oocytes and embryos

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ABSTRACT

The properties of antisense phosphorothioate and unmodified oligodeoxynucleotides have been studied in Xenopus oocytes and embryos. We find that phosphorothioates, like unmodified oligodeoxynucleotides, can degrade Vg1 mRNA in oocytes via an endogenous RNase H-like activity. In oocytes, phosphorothioate oligodeoxynucleotides are more stable than unmodified oligodeoxynucleotides and are more effective in degrading Vg1 mRNA. In embryos, neither unmodified nor phosphorothioate deoxyoligonuleotides were effective in degrading Vg1 message at sub-toxic doses.

INTRODUCTION

Antisense strategies provide a means to suppress a gene's activity and thus deduce its function by examining the resulting phenotype (1,2). Our interest has been in studying the functions of maternal genes in Xenopus development. Maternally transcribed mRNAs, accumulated during oogenesis, guide development until zygotic gene transcription begins at the midblastula transition. In Xenopus, antisense strategies provide one of the few potential methods of determining gene function because mutational analysis is not possible. Additionally, frog embryos and oocytes provide a useful model system in which to study the properties of antisense molecules.

We wish to determine the function of maternally encoded gene products, such as Vg1 protein. Vg1 protein is encoded by a maternal localized transcript which is homologous to TGF-β (3,4,5). Initially, we attempted to inactivate Vg1 by injecting antisense RNA. However, injections of *in vitro* transcribed RNAs antisense to Vg1 into embryos have not resulted in any phenotypes (unpublished data). Our lack of success with antisense RNA against Vg1 led us to explore antisense oligodeoxynucleotides (ODNs) as an alternative approach.

Antisense ODNs have been proposed as therapeutic agents with potential specificity against pathogens and oncogenes. Antisense ODNs were used to inhibit virus replication in cell culture as early as 1978 (6). More recently, it was shown that nuclease resistant methylphosphonate antisense ODNs could inhibit HSV replication in cell culture (7). In cell culture, antisense unmodified ODNs and phosphorothioate ODNs (S-ODNs) have been reported to reduce c-myc protein accumulation (8,9) and to inhibit HIV replication (10,11,12,13). Despite these encouraging results, in no case has the mechanism of action (e.g. RNase H mediated cleavage of message, steric block of translation, disruption of transcript processing) of antisense ODNs been directly demonstrated in tissue culture. It may be difficult to study the mechanism of action of antisense ODNs in tissue culture, due to uncertainties about RNA turnover and delivery of ODNs to cells. In oocytes and embryos, however, the slow turnover of maternal messages and the ability to inject samples into large quantities of cytoplasm make it easier to study the *in vivo* properties of antisense ODNs.

It is important to distinguish between experiments performed in oocytes and in embryos. Oocytes are nondividing immature eggs which are surgically removed from the ovaries of the female. Embryos are fertilized eggs which go through rapid cleavage divisions. In oocytes, unmodified antisense ODNs have been shown to specifically cleave target mRNAs via an endogenous RNase H-like activity (14,15,16,17,18,19). RNase H-like activity has also been detected in S100 extracts of early embryos (20).

In this paper we report that a dose of unmodified ODN greater than 15ng injected into fertilized eggs results in non-specific toxicity: a high proportion of embryos shows aberrant development, even with noncomplementary control sequences. In previous studies, the dose of normal antisense ODNs needed to degrade most of an endogenous target RNA in oocytes and unfertilized eggs is >20ng (15,18,21). Thus, the non-specific toxicity of unmodified ODNs limits their direct microinjection into embryos as an assay for gene function in embryos.

In order to determine why such a large molar excess (>10,000 fold) and high absolute concentration (20ng per embryo ~4μM) is necessary to degrade a message, we performed stability studies with unmodified ODNs. We found by using internally labeled unmodified ODNs, and others have inferred by using end-labeled ODNs, that unmodified ODNs are rapidly degraded in oocytes (16,18). We report in this paper that unmodified ODNs are also very unstable in embryos.

In principle, chemically modified nuclease resistant ODNs should be more stable, and might therefore result in substantial target RNA cleavage at sub-toxic doses. The two most widely

available modified ODNs are methylphosphonates and phosphorothioates (S-ODNs) (7,11). Hybrids of methylphosphonate DNA and RNA are not substrates for RNase H and methylphosphonate ODNs are reported to have low solubility (10,22). We chose to work with S-ODNs because they are more soluble than methylphosphonates, and because, unlike methylphosphonates, they are known to be substrates for bacterial RNase H (24,25).

In this paper, we compare the properties of S-ODNs with unmodified ODNs in both oocytes and embryos. Although S-ODNs are resistant to many nucleases *in vitro* (10,25), their stability *in vivo* has not been reported. Here we show that in oocytes S-ODNs are more stable than normal ODNs. Furthermore, S-ODNs degrade messages via an endogenous RNase H-like activity in oocytes. Presumably because of their enhanced stability, S-ODNs are more effective than unmodified ODNs at degrading Vg1 message in oocytes. In embryos, neither S-ODNs nor unmodified ODNs were effective in degrading Vg1 message at sub-toxic doses. These results are discussed with reference to the potential therapeutic uses of S-ODNs and the use of antisense ODNs to study early Xenopus development.

MATERIALS AND METHODS

Oligodeoxynucleotide synthesis and purification

Oligodeoxynucleotides were synthesized at Gilead Sciences Inc. with standard H-phosphonate chemistry (26,27). The phosphorothioate linkages were prepared as previously described (26,27,28). All ODNs were purified by reverse phase HPLC. A Hamilton polymeric reverse phase HPLC column was used with triethylammonium phosphate buffered (pH6, 50mM) 5% acetonitrile as solvent 'A' and triethylammonium phosphate buffered (50mM, pH8) 75% acetonitrile as buffer 'B'. The ODNs used in antisense and toxicity experiments were additionally purified by a method communicated to us by Doug DeSimone (University of Virginia) which is meant to remove positively charged contaminants. This method involves washing a 5ml Dowex® AG-50−150 cation exchange column with 1M potassium hydroxide until the eluent is pH13. The column is then rinsed with water until the pH of the eluent is neutral. The ODN is then applied to the column. The fractions containing the ODN are then spun through a one ml Sephadex® G50 (equilibrated with distilled deionized water) spin column and concentrated by desiccation (29). ODN concentrations were determined by UV absorbance, assuming 1 OD unit = 37 micrograms per ml. The sequences of the ODN directed against the coding region of Vg1 and the control ODN which is not complementary to Vg1 are shown in figure 1.

Oocyte and embryo treatment

Oocytes and embryos were obtained and treated as previously described (30,2). They were injected with 20−40nl at 0.03−1 micrograms ODN per microliter. We estimate that our dosage measurements are accurate to +/−30%.

RNA isolation and northern blot analysis

Four to ten oocytes or embryos were homogenized in 0.4 mls of homogenization buffer with proteinase K, incubated for 30−60 min. at 37−50°C., phenol/chloroform extracted twice, ethanol precipitated and LiCl precipitated (30). The samples were fractionated on 1% agarose gels, blotted, and hybridized as previously described (29), with ^{32}P labeled UTP RNA probes (31).

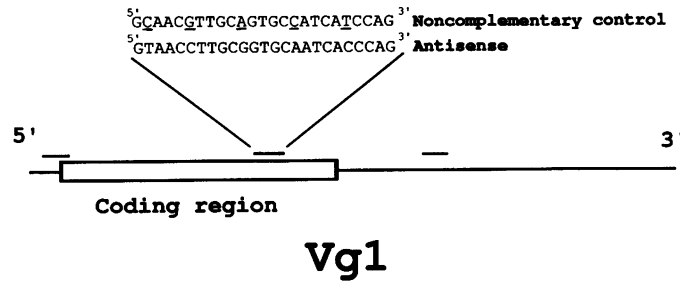

Figure 1. Target sites used in the Vg1 message. The sequence of the ODN directed against the coding region (nucleotides 856−881, ref. 36) of Vg1 is shown. Above this sequence is the noncomplementary control sequence. The order of the underlined bases in the control sequence is different from the antisense sequence. The sequences of the ODNs directed against the 5' and 3' regions of Vg1 mRNA are 5-'CATACTGACAAGCTAGTCTCTGCAG (nucleotides 7−33) and 5'-TCAAGATACTCTGTGCATGTACAGG (nucleotides 1436−1461).

ODN internal labeling

Phosphorothioate ODNs were internally labeled with ^{35}S (32). Internally labeled unmodified ODN was produced using a 7mer primer (5'GGTGATT) and the ODN antisense to the Vg1 coding region (see fig. 1) as template to synthesize a ^{32}P (dATP) labeled 2nd strand with klenow (29). The primer was designed so that the labeled strand would be smaller than the template. The labeled ODN was separated from unlabeled template by denaturing PAGE and isolated by elution and ethanol precipitation (29).

Stability experiments

Internally labeled ODN (5ng−100ng) was injected into oocytes or embryos. The oocytes or embryos were rinsed before homogenization, to remove any uninjected ODN. The ODN was isolated as described for RNA, except that the ODN was ethanol precipitated from 0.3 M Sodium Acetate,10mM $MgCl_2$ with two volumes of ethanol. The LiCl precipitation was omitted because of potential low efficiency of ODN precipitation. The gels used for the S-ODN stability experiment were fixed briefly in 10% acetic acid/ 40% methanol, then treated with Amersham Amplify® before autoradiography.

RESULTS

Unmodified antisense ODNs in fertilized eggs

Our initial objective was to degrade Vg1 mRNA via an RNase H mediated cleavage by injecting antisense ODNs into fertilized eggs. Figure 1 shows the sequence of the antisense ODN targeted against the Vg1 coding region along with the control sequence of identical length and base composition. We have confirmed that the antisense, but not control, ODNs produce the predicted cleavage fragments with SP6 transcribed Vg1 RNA and *E. coli* RNase H *in vitro* (data not shown). Figure 2A shows a northern blot analysis of an experiment in which unmodified ODN antisense to the Vg1 coding region was injected into embryos at 1ng and 50ng doses. A 1ng dose of antisense ODN is not sufficient to degrade Vg1 RNA (figure 2A). With 50ng ODN, Vg1 mRNA was degraded relative to the water control injections. Similar results have been reported by Shuttleworth and Colman (15). However, embryos injected with 50ng became sick and died before gastrulation. In order to test whether this toxicity was due to specific elimination of Vg1 mRNA or due to nonspecific ODN

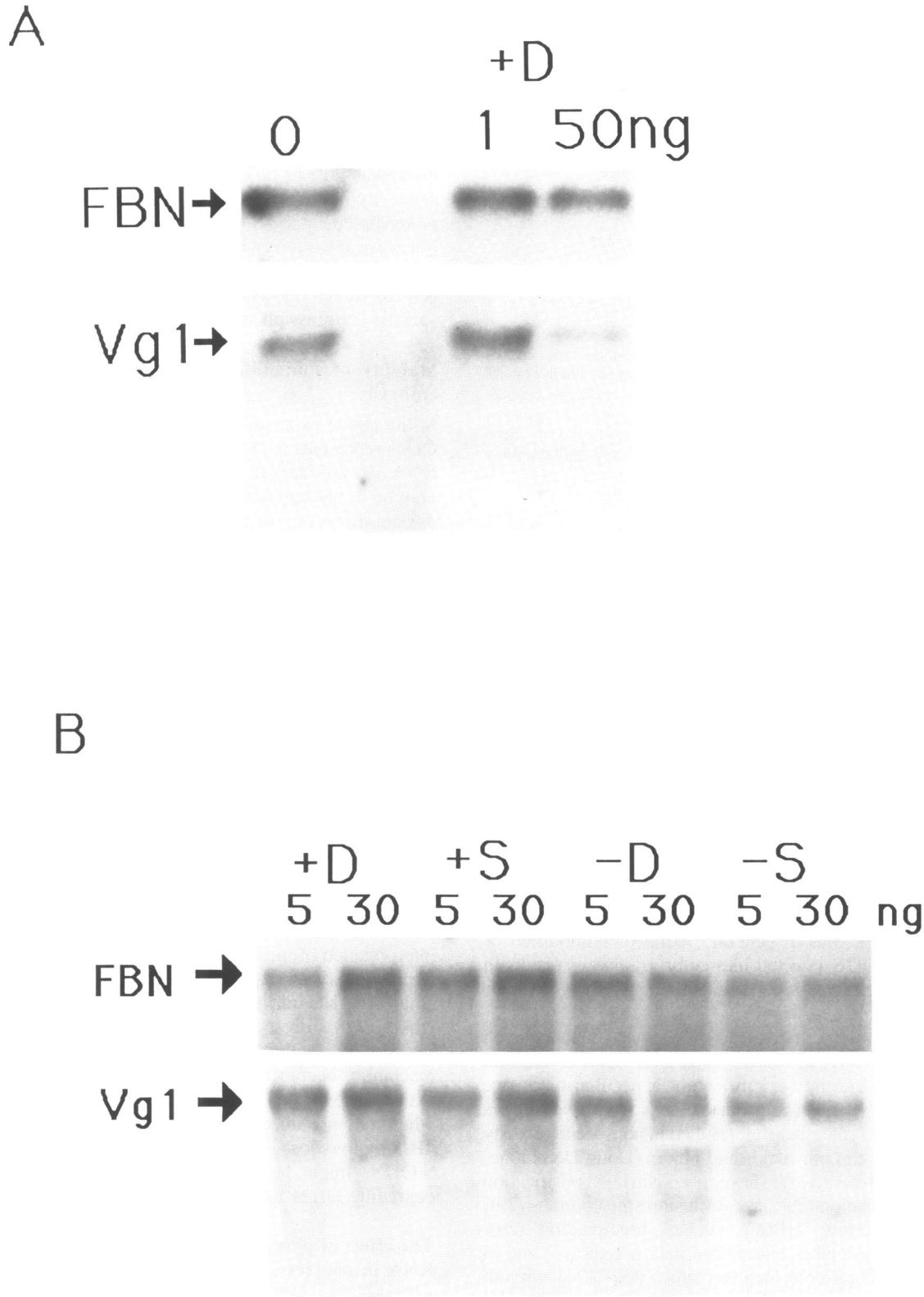

Figure 2. Northern blots of RNA extracted from embryos injected with normal and phosphorothioate oligodeoxynucleotides. Panel A shows an experiment in which only unmodified ODNs were injected. Panel B shows a comparison of unmodified and phosphorothioate ODNs. Unmodified (D for diester) or phosphorothioate (S for sulfur) ODNs were injected into fertilized embryos at the doses shown. RNA was extracted after 3 hours, fractionated, blotted, and probed with Vg1 and with fibronectin (FBN) as a control for RNA recovery. In the experimental lanes, marked '+', a 25mer ODN antisense to the coding region (sequence shown in figure 1) was injected. In the negative control lanes, marked '−', a 25mer with a noncomplementary sequence of identical length and base composition was injected.

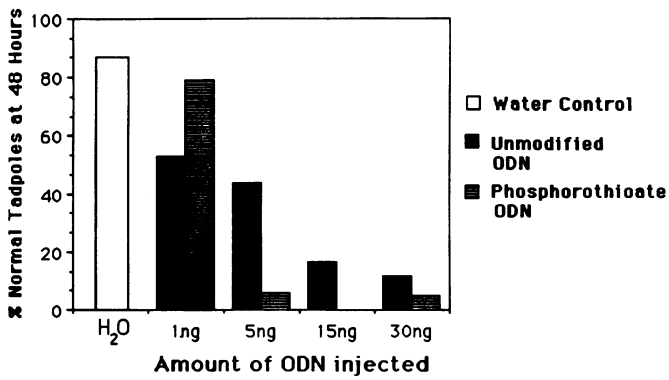

Figure 3. Toxicity of unmodified and phosphorothioate oligodeoxynucleotides in embryos. Unmodified or phosphorothioate noncomplementary control ODNs (sequence shown in figure 1) were purified by HPLC, ion exchange, and size exclusion columns. They were then injected into fertilized eggs at the doses shown. The embryos were scored 48 hours later for normal external morphology. Each bar represents at least 17 embryos. All embryos were from a single fertilization of eggs from a single female.

toxicity, we tested the toxicity of noncomplementary control ODNs.

The toxicity of ODNs was tested by injecting various doses of the control noncomplementary ODNs into fertilized eggs and following their development into tadpoles. The 'non-specific' ODN used in figure 3 might hybridize to some required message by chance; however, the non-specific toxicity has been observed with several different ODN sequences. As shown in figure 3, 90% of the embryos injected with 30ng of noncomplementary unmodified ODN or S-ODN are dead or have developed abnormally by 48 hours post injection. At doses of 30ng, the nonspecific toxic effects of ODNs become apparent several hours after injection. There is a visible slowing of the cell cycle in the blastomeres nearest the injection site. In extreme cases, cell lysis occurs as early as 6 hours after injection. The results in figure 3 are from one fertilization and are typical. The exact dose required to give non-specific toxic effects varies with each injection experiment, but in general, 30 ng of unmodified or phosphorothioate ODN injected into embryos always results in non-specific toxic effects, while 1ng consistently shows little or no toxic effects.

The toxicity we observe may be due to chemical contamination arising during the ODN synthesis. This is difficult to rule out completely; however, the toxicity is not a peculiarity of one particular chemical synthesis, because ODNs from five sources using various ODN synthesizers all resulted in nonspecific toxicity at doses above 20ng in our hands and others (Doug DeSimone, pers. comm.) Moreover, we have tried various purification schemes (gel purification (29), size exclusion spin columns (29), ion exchange columns, HPLC, ethanol precipitation (29), extensive dialysis and ether extraction; data not shown) and in no case have we been able to inject more than 20ng without producing non-specific toxic effects in most of the embryos. We therefore suspect that the toxicity is inherent to the ODN itself or its degradation products.

Phosphorothioate and unmodified ODNs are ineffective at sub-toxic doses in embryos

To determine if specific degradation of Vg1 mRNA could occur at sub-toxic doses, we injected intermediate doses of unmodified and phosphorothioate ODN into embryos. The lanes marked 'D+' in figure 2B show the northern blot analysis of fertilized eggs injected with a 5 or 30 ng dose of unmodified ODN complementary to the Vg1 coding sequence. It can be seen that little degradation of Vg1 occurred by comparing the Vg1 signal in the antisense lanes (D+) to the control lanes (D−) in which a noncomplementary ODN was injected at the same doses. In this experiment we also used phosphorothioate ODNs (S-ODNs). However, little or no degradation of Vg1 occurred, as can been seen by comparison to the control lanes in which noncomplementary S-ODN was injected (lanes S+ and S−). Since we have never observed substantial degradation of Vg1 mRNA with sub-toxic doses of unmodified or S-ODNs, we believe direct injection into embryos is unlikely to result in specific antisense phenotypes for Vg1 or other maternal RNAs.

Stability of unmodified and modified ODNs in eggs and embryos

In the above experiments we estimate that a 30ng injection of ODN represents a >10,000 fold molar excess over its target RNA and an internal concentration of ~4μM (assuming a cell volume of one microliter). To investigate why such large doses did not efficiently degrade Vg1, we injected internally labeled ODNs into oocytes and embryos, and examined their stability by PAGE. The use of internally labeled ODNs is important in order to distinguish between complete degradation and phosphatase or minor exonuclease activity. The results with unmodified ODNs are shown in figure 4A. In both oocytes and embryos, unmodified ODNs are rapidly degraded, with a half-life of less than 30 minutes. Although there are slight variations in the apparent degradation rate in each experiment, the experiments have been repeated three times and the half-life of unmodified ODNs is always less than 30 minutes. Some of the radioactive signal lost from the ODN position on the gels appears as higher molecular weight material, which is not shown in figure 4A. The results in oocytes are consistent with other reports using end-labeled ODNs (16,18).

The stability of internally labeled S-ODNs was also tested. In embryos, internally labeled S-ODNs injected at low doses (<5ng) have a half-life of less than 30 minutes (fig. 4B). However, the interpretation of this experiment is complicated by the result that larger doses (~30ng) of S-ODN appear to be more stable in embryos (data not shown). The larger dose also makes the embryos visibly sick, which adds to the difficulty of interpretation. One possible explanation is that the degradation machinery becomes saturated at higher doses.

In oocytes S-ODNs have a half-life of greater than 3 hours when they are injected with either a high dose (~100ng, fig. 4B) or a low dose (<5ng; data not shown). This suggested that S-ODNs might be more effective than unmodified ODNs at degrading target mRNAs in oocytes.

The effect of phosphorothioate vs. unmodified ODNs on Vg1 RNA in oocytes

Various amounts of unmodified and phosphorothioate antisense ODNs were injected into stage V-VI oocytes, and total RNA was extracted after 5 hours for northern blot analysis. A 30ng dose of the S-ODN in oocytes was more effective in degrading Vg1 message than an unmodified ODN of identical sequence (fig. 5). The degradation caused by either 15ng or 30ng of normal ODN was only ~40% relative to control injections of noncomplementary ODNs. The degradation with the S-ODN was 40% and 70% with 15ng and 30ng injections respectively (as

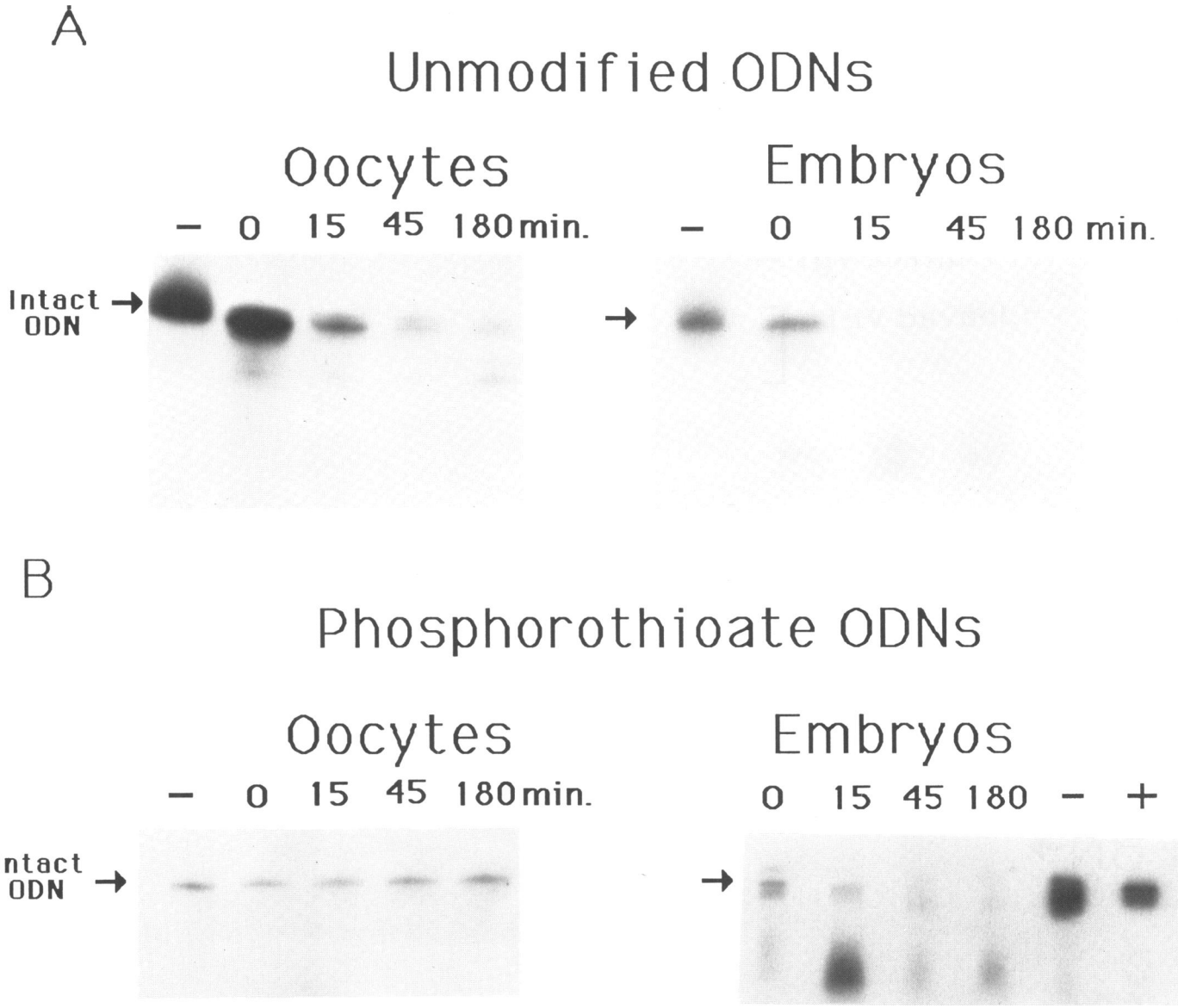

Figure 4. Stability time courses of internally labeled unmodified and phosphorothioate oligodeoxynucleotides in oocytes and embryos. Internally labeled unmodified (Panel A) or S-ODNs (panel B) were injected into embryos or oocytes. In each experiment the ODNs were extracted and analyzed by PAGE and autoradiography. The additional control lane in panel B marked '+' is the S-ODN used in this experiment incubated with DNase I *in vitro*. Note that, as described in the text, S-ODN stability in embryos may be dependent on the dose injected.

determined by scanning densitometry). The degradation by S-ODNs was deemed specific by three criteria. A control endogenous RNA (fibronectin) was not degraded by ODN directed against Vg1, which shows that the effect is not due to nonspecific degradation of all RNAs. The injections with control S-ODNs of identical length and base composition at the same concentration produced no effect, which demonstrates that the degradation is dependent on the sequence being antisense. Finally the presence of the 3′ degradation fragment of the predicted size demonstrates that the S-ODN acts to specifically cleave the message by an endogenous RNase H-like activity. Additional evidence for specific degradation by an endogenous RNase H-like activity is shown in the lanes marked 'M' in figure 4. This shows RNA from oocytes that had been injected with a mixture of three antisense S-ODNs targeted to different regions of the Vg1 message (see figure 1 for description of these ODNs). The additional cleavage fragment in this lane corresponds to the predicted 3′ degradation product of one of the additional target sites. The blot was stripped and probed with a 3′ specific Vg1 probe to confirm that the band was actually the 3′ degradation fragment (data not shown).

Although the above results were encouraging, the amount of S-ODN necessary to substantially degrade message was still greater than the amount of unmodified ODN reported to be necessary to degrade other endogenous messages (15,18). We therefore attempted to exploit the enhanced stability of S-ODNs by injecting lower doses of S-ODNs and incubating the oocytes for longer periods of time. Five nanograms of unmodified or S-ODNs were injected into oocytes and RNA was extracted at 0, 6 and 24 hours and analyzed by northern blot, as shown in figure 6. The five nanogram doses of both unmodified and S-ODNs show some specific degradation of Vg1 message at 6 hours, but

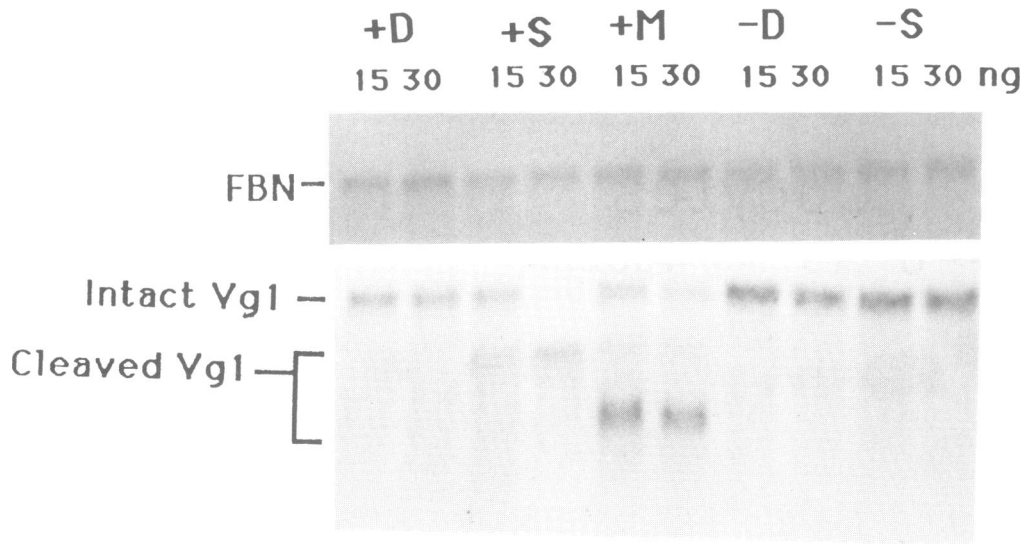

Figure 5. Effectiveness of a phosphorothioate vs. unmodified ODN in oocytes. Unmodified diester (D) or phosphorothioate (S) ODNs were injected into stage V-VI oocytes at the doses shown. RNA was extracted after six hours, fractionated, blotted, and probed with Vg1 and fibronectin (FBN) as a control for RNA recovery. In the experimental lanes (+D, +S), 25mer antisense ODNs against the coding region of Vg1 were injected (sequence shown in figure 1). In the negative control lanes (−), ODNs with a noncomplementary sequence of identical length and base composition were injected. In the lanes marked '+M' a mixture of three antisense S-ODNs complementary to different regions of Vg1 mRNA were injected at the total dose shown.

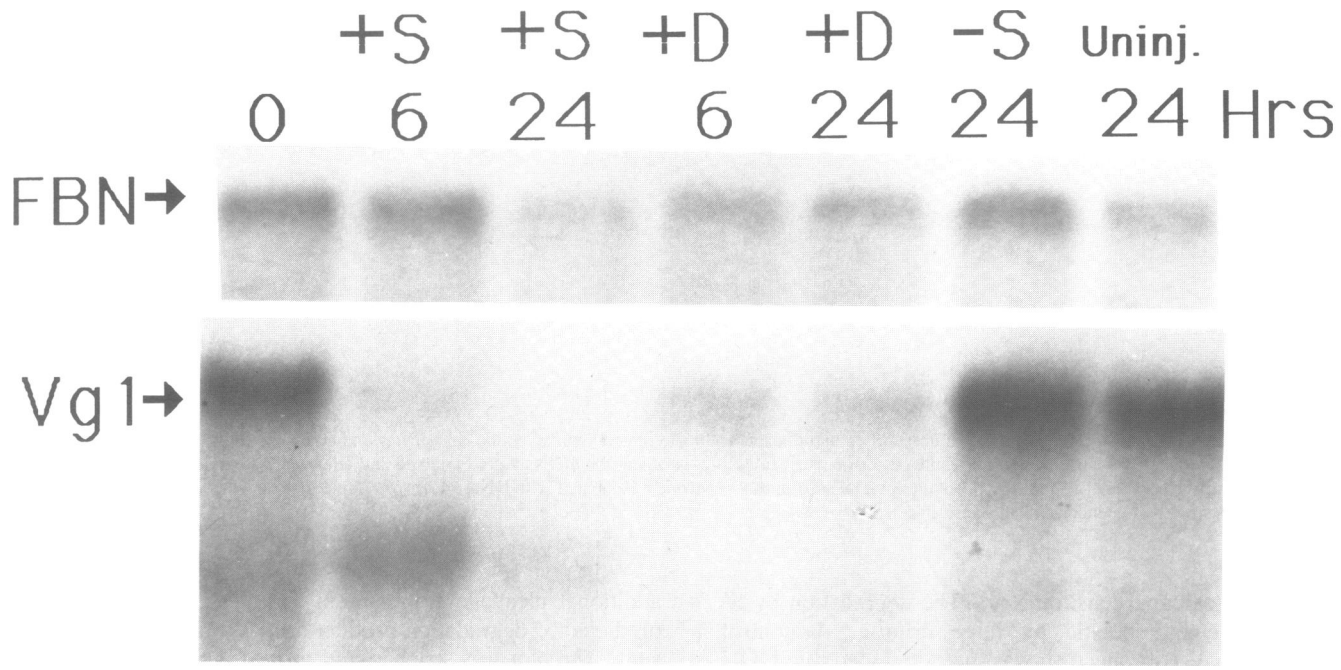

Figure 6. The effectiveness of low doses of phosphorothioate oligodeoxynucleotide in degrading Vg1 in oocytes. Five nanogram doses of normal diester (D) or phosphorothioate (S) ODNs were injected into stage V-VI oocytes. RNA was extracted at the times shown, fractionated, blotted, and probed with Vg1 and fibronectin (FBN) as a control for RNA recovery. In the experimental lanes (+), 25mer antisense ODNs against the coding region of Vg1 were injected (sequence shown in figure 1). In the phosphorothioate negative control lane (−), ODNs with a noncomplementary sequence of identical length and base composition were injected and incubated for 24 hours. The other negative control lane (uninj.) contains RNA from uninjected oocytes incubated for 24 hours.

only the S-ODN continues to degrade Vg1 up to 24 hours. By scanning the films, using fibronectin as an internal standard, we determined that the S-ODN degraded >80% of Vg1 message after 24 hours. Uninjected oocytes cultured for 24 hours or oocytes injected with 5ng of noncomplementary control S-ODN showed no degradation of Vg1. The result that a 5ng dose of antisense S-ODN degrades > 80% of Vg1 with a 24 hour incubation in oocytes has been repeated in three separate experiments. We have no evidence of nonspecific toxicity to the oocyte with a 5ng dose of S-ODN; 24 hours after injection RNAs not targeted remain intact (see fibronectin control, fig. 6), and there is no external sign of toxicity.

DISCUSSION

In order to determine the function of Vg1, we have tried to cleave the message via endogenous RNase H by injecting antisense ODNs. In embryos, unmodified ODNs injected at the maximal nontoxic dose were not sufficient for substantial cleavage of Vg1 mRNA. We have found that normal ODNs are very unstable in embryos, so we have turned to phosphorothioate ODNs (S-ODNs), which are resistant to many nucleases. Unfortunately, the S-ODNs were also unstable and ineffective at subtoxic doses when injected into embryos. In oocytes S-ODNs were found to be much more stable than unmodified ODNs. With long incubations, we have been able to degrade more than 80% of Vg1 mRNA using only 5ng of S-ODN. This is roughly an order of magnitude less than the reported doses (30–150ng) of normal ODNs necessary to degrade >80% of Vg1 and other endogenous messages in oocytes (17,18,35).

Direct injection of unmodified or phosphorothioate ODNs into fertilized eggs is unlikely to yield specific antisense phenotypes because of ODN toxicity. An alternative strategy is to inject oocytes with antisense ODNs, degrade the target mRNA, mature the oocytes and reimplant them into a host female to obtain embryos that are not injured by the ODN toxicity (35). We believe that the prospects for doing this successfully with normal ODNs are marginal because large doses are necessary to specifically degrade endogenous messages. Unpublished reports (Doug DeSimone, University of Virginia, pers. comm.) suggest that these high doses injected into oocytes result in non-specific toxic effects in embryos derived from these oocytes. S-ODNs, since they are effective at lower doses in oocytes, might allow the reimplantation strategy to work.

There are several potential difficulties with the reimplantation strategy. If the toxicity to development is proportional to the amount of intact ODN in the oocyte integrated over time, then this approach would be destined to failure; a more stable ODN would necessarily be more toxic. Moreover, even if there is a dose at which specific degradation of target mRNA occurs without nonspecific toxic effects, much caution must be used in interpreting phenotypes. Since we have seen variations in the toxicity of ODNs from different sources, one must be sure to get consistent negative results with several control sequences, and to have positive results with more than one antisense sequence. Rescuing the phenotype by subsequent injection of *in vitro* transcribed message may be necessary to draw a firm conclusion.

Since S-ODNs can be more stable *in vivo* and can degrade messages via endogenous RNase H *in vivo*, they are an attractive tool for antisense studies in other systems. It would be informative to use northern blot analysis to determine whether antisense S-ODNs degrade RNAs in tissue culture by this mechanism. Lastly, with regard to potential therapeutic uses of S-ODNs, since we have evidence of S-ODN degradation, and since phosphorothioate mononucleotides are readily incorporated into DNA by polymerases (34), the possibility of complex side effects due to incorporation into genomic DNA must be addressed.

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